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Original Article

Resveratrol Suppresses Expression of VEGF by Human Retinal Pigment Epithelial Cells: Potential Nutraceutical for Age-related Macular Degeneration

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ABSTRACT: Age-related macular degeneration (AMD) is a sight threating retinal eye disease that affects millions of aging individuals world-wide. Choroid-retinal pigment epithelium (RPE)-neuroretina axis in the posterior compartment of the eye is the primary site of AMD pathology. There are compelling evidence to indicate association of vascular endothelial growth factors (VEGF) to AMD. Here, we report the inhibitory actions of resveratrol (RSV) on inflammatory cytokine, TGF-β and hypoxia induced VEGF secretion by human retinal pigment epithelial cells (HRPE). HRPE cultures prepared from aged human donor eyes were used for the studies in this report. HRPE secreted both VEGF-A and VEGF-C in small quantities constitutively. Stimulation with a mixture of inflammatory cytokines (IFN-γ, TNF-α, IL-1β), significantly increased the secretion of both VEGF-A and VEGF-C. RSV, in a dose dependent (10-50 uM) manner, suppressed VEGF-A and VEGF-C secretion induced by inflammatory cytokines significantly. RT-PCR analysis indicated that effects of RSV on VEGF secretion were possibly due to decreased mRNA levels. TGF-β and cobalt chloride (hypoxia mimic) also upregulated HRPE cell production of VEGF-A, and this was inhibited by RSV. In contrast, RSV had no effect on anti-angiogenic molecules, endostatin and pigment epithelial derived factor secretion. Studies using an in vitro scratch assay revealed that wound closure was also inhibited by RSV. These results demonstrate that RSV can suppress VEGF secretion induced by inflammatory cytokines, TGF-β and hypoxia. Under pathological conditions, over expression of VEGF is known to worsen AMD. Therefore, RSV may be useful as nutraceutical in controlling pathological choroidal neovascularization processes in AMD.

Key words: Resveratrol, VEGF, SIRT1, Cytokines, Retina, Retinal pigment epithelium, Age-related macular degeneration

Age-related macular degeneration (AMD) is a multifactorial disease affecting the retina that may cause vision loss in the aging population [1-4]. AMD primarily affects macular region in the retina that is critical for central vision. Since AMD is a slowly progressing disease taking many years to develop, it is difficult to follow the precise pathophysiological mechanisms causing the disorder [1-5]. However, clinico-pathological observations and epidemiological studies provided valuable insight into understanding the possible pathways leading to AMD. The primary site of AMD is closely associated with choroid- Bruch's membrane (BM)-retinal

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pigment epithelium (RPE)-neuroretina (NR) component in the posterior part of the eye [1, 4, 5]. AMD is generally classified as geographic atrophy (dry AMD) or exudative (wet AMD). Dry AMD is characterized by accumulation of drusen (gluey substance) around RPE and some degree of RPE degeneration [1, 5, 6]. Wet AMD is characterized by the presence of choroidal neovascularization (CNV) in which newly formed blood vessels from choriocapillaris penetrate through BM and RPE into the photoreceptor layer in NR [1,4,5-7]. Wet AMD accounts for less than 10% of AMD, but serious complications arise if untreated leading to complicated retinal disease [1, 4, 7-9].

RPE plays a central role in the pathogenesis of AMD. The RPE is strategically located between choroid and neurosensory retina and serves as the outer retinal barrier to regulate the transport of fluids, nutrients and waste materials [5, 10-12]. RPE synthesizes a number of extracellular matrix (EM) proteins and proteoglycans such as collagens, fibronectin, chondroitin and heparin that are vital for the maintenance of BM and interphotoreceptor matrix (IPM) [10-12]. Another specialized function of RPE is phagocytosis and degradation of outer segments of photoreceptors which are in intimate contact with microvilli [10-12]. In addition, RPE secretes a variety of cytokines, chemokines and growth factors such as IL-6, IL-11, CXCL-9, PDGF, TGF-β and VEGF [13-19]. Overall, healthy RPE is essential for the normal functioning of underlying choroid and overlying neurosensory retina [18-21]. Earlier studies have revealed the presence of VEGF and other angiogenic molecules associated with macrophages, lymphocytes and endothelial cells within CNV membranes [6, 22-25]. Due to the close association of VEGF to CNV, anti-VEGF therapies are the main focus for the management of AMD [26-28].

Vascular endothelial growth factors (VEGFs), protein molecules secreted by a variety of cells, primarily act on endothelial cells to initiate and promote angiogenesis during development and in pathological conditions [29, 30]. Among VEGFs, VEGF-A and VEGF-C are critical in the formation and maintenance of vascular and lymphatic vessels [29-32]. VEGF-A is encoded by a single gene and alternate splicing results in four major mRNA species coding for 121,165,189 and 206aa proteins [29-31]. VEGF-A, but not VEGF-C, gene has hypoxia regulatory element and heparin binding sites [29-32]. VEGF-A acts through VEGF-R1 and VEGF-R2 receptors present on blood vessel endothelial cells while VEGF-C acts through VEGF-R2 and VEGF-R3 present on endothelial cells present in blood and lymphatic vessels [29-33]. VEGFs, also known as vascular permeability factors, alter endothelial tight junctions and allow leakage of fluids from blood vessels during elevated levels of VEGF under certain pathological conditions [29, 30, 34].

Resveratrol (RSV) is a polyphenol phytoalexin that belongs to stilbene (3, 4', 5-trihydroxystilbene) class [35-38]. RSV is present in many fruits and other plant products, but grape skin and seeds appear to be the rich source of RSV [37, 38]. RSV exists in both *cis* and *trans* forms but *trans* form is more stable. RSV is shown to be rapidly absorbed, both in *in vivo* human studies and *in vitro* cell culture studies, and is conjugated to form RSV glucoronide and RSV sulfate [35, 36]. RSV is known as an anti-aging, anti-diabetic, anti-cancer and cardio protective agent and acts by modulating various physiological processes like cell proliferation, apoptosis, inflammation, metastasis and angiogenesis [37-40]. Most of the activities of RSV are mediated through SIRT1 (mammalian orthologue of yeast sir2 (silent information regulator 2)), which in turn acts by deacetylation (histone deacetylase-3) of transcription factors and other cellular proteins [37, 38, 41, 42]. Expression of SIRT1 is critical for many normal developmental and physiological activities, since SIRT1gene knock-out mice die perinatally with defects in retina, bone and heart [43]. Retinal defects include disorganization and reduced thickness of all the layers of neuroretina including retinal pigment epithelium. These results strongly indicate critical role of SIRT1, mediator of RSV, in retinal structure, organization and function.

Our previous studies showed that inflammatory cytokines IFN-γ, TNF-α, IL-1, TGF-β and hypoxia significantly up-regulate gene expression and secretion of VEGF-A and VEGF-C by HRPE cells [14, 18]. Now, we wanted to explore the possible beneficial effects of RSV on the regulation of VEGF expression by HRPE cells. In this report, we show that RSV suppresses VEGF-A and VEGF-C secretion induced by inflammatory cytokines, TGF-β and hypoxia. Since RPE and VEGF are among the major players in the pathology of AMD, our findings indicate that RSV may be useful as a nutraceutical for the alleviation of pathology of CNV in the wet form of AMD.

MATERIALS AND METHODS

Reagents

Resveratrol was purchased from Sigma Chemical, Saint Louis, MO. Human rTNF-α and rIFN-γ were obtained from Roche Applied Science, Indianapolis, IN. Human VEGF-C and endostatin Elisa kits, and rIL-1β and other cytokines were obtained from R&D Systems, Minneapolis, MN. Human VEGF-A ELISA kits were purchased from Invitrogen, Carlsbad, CA. PEDF (pigment epithelium-derived factor) ELISA kits were obtained from BioProducts MD, Walkersville, MD. RNA PCR kit and other PCR reagents were obtained from Applied Biosystems, Foster city, CA. Fetal bovine serum

and cell culture media were obtained from Invitrogen Corporation, Carlsbad, CA. CellTiter 96 Aqueous One solution cell proliferation assay kit was obtained from Promega, Madison, WI.

HRPE Cell Cultures

Primary cultures of human retinal pigment epithelial cells (HRPE) were prepared from adult donor (70-90 years) eyes as described previously [13, 14, 18]. HRPE cultures were grown in minimum essential medium supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (25 ng/ml). Positive immunostaining of all the cells with cytokeratin antibody confirmed the epithelial phenotype of our HRPE cells excluding the presence of non-epithelial cell types, such as fibroblasts, endothelial and/or smooth muscle cells, present in the retina and the choroid. HRPE cultures at passages 8 to 12 were used in this study.

Analysis of secreted VEGF-A, VEGF-C and other proteins by ELISA

HRPE cultures were grown to confluence in 24 well culture plates in 10% FBS containing media. Cultures were washed twice with serum-free medium (SFM) and the indicated reagents prepared in SFM were added (1 ml/well). After 24h, culture supernatant fluids were collected and frozen for later analysis. To avoid the effects and interactions of various growth factors, cytokines and other proteins present in the serum, all treatments were performed in SFM. Culture supernatants were clarified by centrifugation for 2 min in a microfuge at 14 k rpm. Levels of secreted VEGF-A, VEGF-C, PEDF and endostatin proteins were determined by specific immunoassays following the manufacturer's instructions.

HRPE cell viability in the presence of resveratrol and inflammatory cytokines

HRPE cultures were grown to confluence in 96 well culture plates in 10% serum containing media. Phenol Red free MEM was used in all the subsequent steps to avoid color interference from Phenol Red present in the tissue culture medium. Then cultures were washed with SFM once and left in SFM for 3-4 h. Media were removed from the wells and SFM containing control (DMSO control) or different concentrations (2 to 100 uM) of RSV (dissolved in DMSO) was added to the wells. After incubating cells for 24h, 20 ul of CellTiter Aqueous One reagent (Promega) was added and further incubated for 1 to 2h till the optimum color was developed. These experiments were also performed in the presence of an inflammatory cytokine mixture (IFN-γ, 20U/m+ TNF-α, 2 ng/ml+ IL-1β, 2ng/ml) and various concentrations of RSV. The color developed due to the formation of soluble formazan produced by cellular reduction of tetrazolium compound was read at 490nm in a 96 well plate reader. The optical density values are proportional to the viability of the cells in each well. For each treatment, 8 wells were used and means and SEM were calculated. Results are from one typical experiment representative of two other experiments.

RT-PCR analysis of VEGF-A and VEGF-C mRNA in HRPE cultures

HRPE cultures were grown to confluence in 60 mm culture dishes in media containing 10% FBS. Cultures were washed with SFM twice and incubated in SFM containing inflammatory cytokine mix (ICM) containing IFN-γ (20 U/ml), TNF-α (2 ng/ml) and IL-1β (2 ng/ml) for 8h in the presence of varying concentrations of RSV. Total RNA from cultures was prepared by using RNA Stat-60 extraction solution (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. The following primers were used: VEGF-A forward 5'- CCA TGA ACT TTC TGC TGT CTT- 3', VEGF-A reverse 5'- TCG ATC GTT CTG TAT CAG TCT-3' (PCR product size= 516, 648, 720, 771 bp); VEGF-C forward 5'- GTC TGT GTC CAG TGT AGA TG- 3', VEGF-C reverse 5'- AGG TAG CTC GTG CTG GTG TT-3' (PCR product size, 360 bp); GAPDH forward 5'- CCA CCC ATG GCA AAT TCC ATG GCA – 3', GAPDH reverse 5'- TCT AGA CGG CAG GTC AGG TCC ACC - 3'(PCR product size, 600 bp). RT-PCR was performed following the protocol provided by the manufacturer and as described earlier [14, 18]. PCR products were separated by electrophoresis in 4% Nusieve 3:1 Plus agarose gel containing ethidium bromide (Lonza, Rockland, ME), photographed and integrated using Kodak Gel Logic 200 imaging system.

Effect of resveratrol on HRPE culture wound closure by in vitro scratch assay

HRPE cultures were grown to confluence in 35 mm Primaria tissue culture dishes and 8 well tissue culture treated slides (Nalge Nunc, Rochester, NY). Using 10 ul ultramicrotip (Eppendorf), cultures were scraped off from one side to the other side carefully in the center of the well or slide [15]. This scratching makes a gap of cell layer of approximately similar width that is devoid of cells. Cultures were washed with SFM twice to remove detached and floating cells and cellular debris. Then 5% FBS media alone or containing various concentrations of resveratrol were added to the designated dishes or wells

in the slides. Similar studies were also conducted in SFM in the presence of RSV. After 48 h incubation in tissue culture incubator, cells were fixed with ethanol and stained with GIEMSA stain (Karyomix, GIBCO). Cell migration was observed in live cultures and stained cells were photographed.

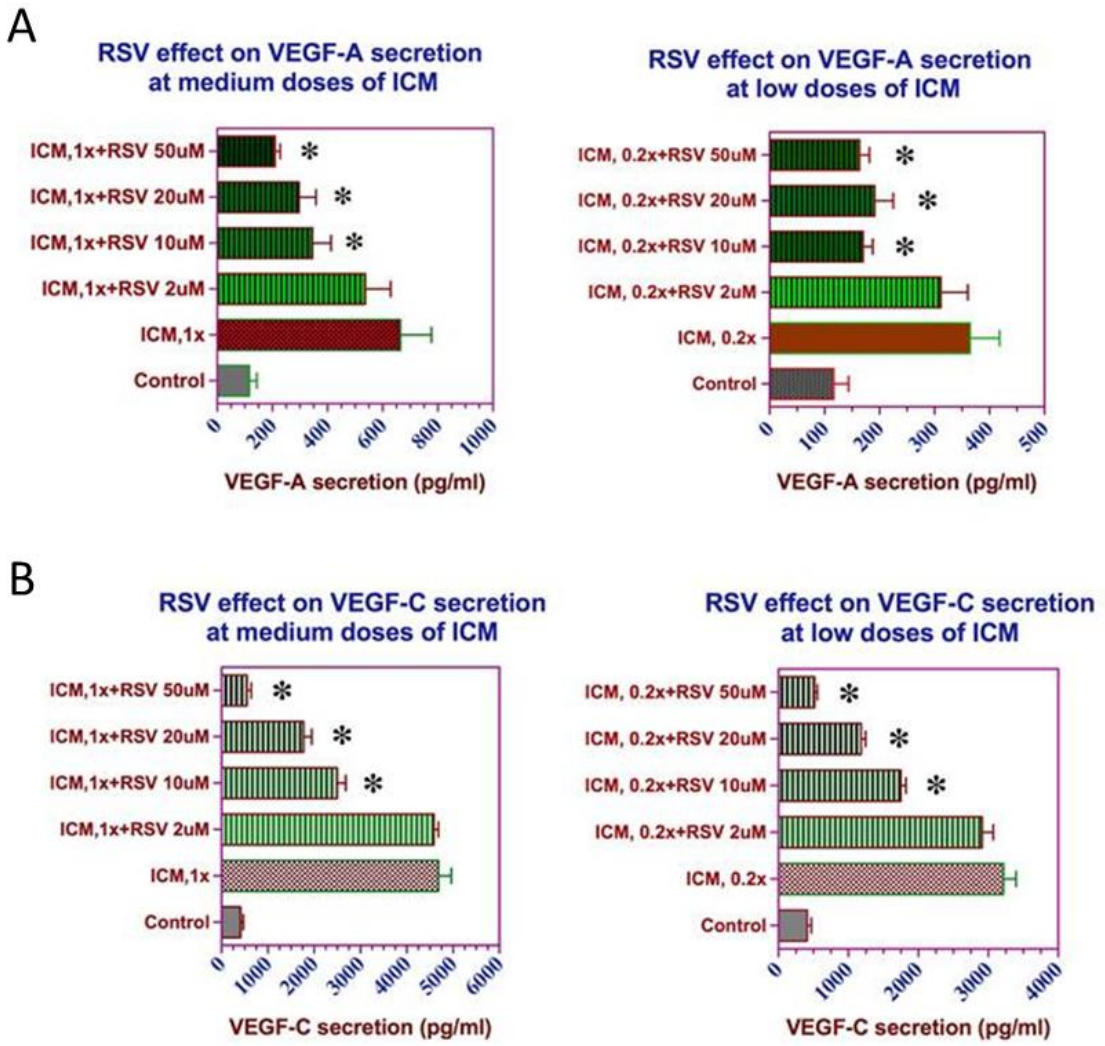

Figure 1. Effect of RSV on VEGF secretion by HRPE cells. Resveratrol inhibits VEGF-A **(A)** and VEGF-C **(B)** secretion by HRPE cells at medium and low concentrations of ICM. HRPE cultures grown to confluence in 24 well plates were treated with ICM 1x ((IFN-γ 10U +TNF-α 1ng + IL-1β 1ng/ml) or ICM 0.2x ((IFN-γ 2U +TNF-α 0.2ng + IL-1β 0.2ng/ml) in the presence of RSV (2-50 uM) in serum free medium for 24h. Then culture supernatant fluids were collected and the levels of VEGF-A and VEGF-C determined by ELISA. Results are means ± SEM of 4 experiments each with duplicate samples. Effect of RSV compared to ICM alone, *p values < 0.01-0.001.

RESULTS

Our previous studies have shown that inflammatory cytokines (IFN-γ, TNF-α and IL-1β) significantly enhance VEGF-A and VEGF-C expression by human retinal pigment epithelial cells [18]. Initial studies were conducted to examine the effects of RSV on ICM 10x (IFN-γ 100 U+TNF-α 10 ng+IL-1β10 ng/ml) induced VEGF-A and VEGF-C secretion by HRPE cells (supplement Fig. A). Inverted microscopic observations of HRPE cells did not reveal any toxicity under these treatment conditions. ICM 10x induced secretion of both VEGF-A and VEGF-C was significantly down regulated by RSV at concentrations of 10, 20, and 50uM. RSV effect on VEGF-C secretion was more pronounced than on VEGF-A secretion. In all further studies, we used lower concentrations of inflammatory cytokines.

To determine the possible toxicity of ICM or RSV alone and together, we performed cell viability tests using Cell Titer Aqueous One reagent (PROMEGA). HRPE cultures were exposed to RSV alone (2 -100 uM) or in the presence of ICM 2x (20 U IFN-γ + 2 ng TNF-α + 2 ng IL-1β/ml). After 24 h incubation, cell viability was tested and expressed as arbitrary optical density units. RSV alone or RSV+ICM treatment did not exhibit any significant effects on HRPE cell viability (supplement Fig. B).

Next, we examined the effects of RSV on VEGF-A secretion induced by lower concentrations of ICM. ICM 1x (10 U IFN-γ + 1 ng TNF-α + 1 ng IL-1β/ml) and ICM 0.2x (2 U IFN-γ + 0.2 ng TNF-α + 0.2 ng IL-1β/ ml) significantly enhanced VEGF-A secretion by HRPE cells (Fig. 1A). RSV (2 uM) had no significant influence on ICM enhanced VEGF-A secretion. Higher concentrations of RSV (10-50 uM) inhibited VEGF-A secretion by HRPE significantly at both ICM 1x and ICM 0.2 x (Fig. 1A). Secretion of VEGF-C was also significantly enhanced in the presence of ICM 1x and ICM 0.2x by HRPE cells (Fig. 1B). RSV (10-50 uM) significantly inhibited ICM induced VEGF-C secretion (Fig. 1B). Under various conditions HRPE consistently secreted higher levels of VEGF-C in comparison to VEGF-A (Fig. 1A & B).

RT-PCR analysis of VEGF-A and VEGF-C gene expression was performed to correlate secretion of VEGF protein to the mRNA levels. Under control conditions, very faint bands of VEGF-A and VEGF-C PCR products are seen (Fig. 2, lane1). In the presence of ICM, VEGF-A and VEGF-C PCR products exhibited intense bands (Fig. 2, lane2). VEGF-A and VEGF-C PCR products band intensities are clearly reduced in the presence of RSV in ICM treated HRPE cells (Fig. 2 lanes 3-6). Treatment of HRPE cells with media or RSV alone (lane 1 and lane 7) yielded similar band intensities suggesting that RSV had no effect on constitutively expressed VEGF by HRPE cells.

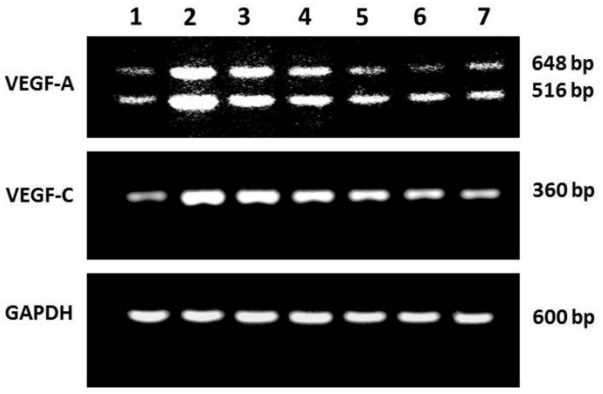

Figure 2. Resveratrol inhibits inflammatory cytokine mix (ICM) enhanced VEGF-A and VEGF-C mRNA expression by HRPE cells. Confluent cultures of HRPE grown in 60 mm plates were treated with ICM 2x (20u IFN-γ+2 ng TNF-α +2 ng IL-1β/ml) in the presence of various concentrations (2-50 uM) of RSV for 8h in SFM. Total RNA prepared was used for RT-PCR analysis as described in the methods section. PCR amplification cycles were 25 for GAPDH and 30 for VEGF-A and VEGF-C. PCR products for VEGF-A, 516bp and 648 bp, represent VEGF-A proteins 121aa and 165aa respectively. PCR products in each group were exposed and integrated to the same intensity. Therefore comparisons of the band intensities between the groups are not appropriate. 1=control, 2=ICM 2x, 3=ICM 2x+RSV (2 uM), ICM 2x+RSV (10 uM), 5= ICM 2x+RSV (20 uM), 6=ICM 2x+RSV (50 uM), 7=Control+RSV (20 uM)

Our previous studies demonstrated that TGF-β and CoCl2 (hypoxia mimic) enhances VEGF-A secretion by HRPE cells [14]. Therefore, it is of interest to know if RSV suppresses TGF-β and CoCl2 induced VEGF-A secretion. HRPE cultures grown in 24 well plates were treated with ICM 2x (20 U IFN-γ +2 ng TNF-α + 2 ng IL-1β/ml), TGF-β1 (2 ng/ml) or CoCl2 (200 uM) alone and in the presence of RSV (10-50 uM). ICM 2x, TGF-β1 or CoCl2 enhanced VEGF-A secretion was significantly inhibited by RSV (Fig. 3A). We also analyzed these treatment samples for secreted VEGF-C. ICM induced VEGF-C secretion was significantly suppressed by RSV (Fig. 3B). However, TGF-β1 or CoCl2 did not influence VEGF-C secretion under these conditions and RSV had no effect on VEGF-C levels (Fig. 3B). RT-PCR analysis was performed to confirm the effects of TGF-β1 on VEGF-A and VEGF-C mRNA levels (Fig. 4). PCR product band for VEGF-A was more intense in TGF-β 2x treated (lane 2) samples compared controls (lane 1, upper panel). A dose dependent decrease in band intensities was

observed in RSV treated HRPE cells (Fig. 4, upper panel, lane 3-6). VEGF-C mRNA levels were not altered by TGF-β treatment as indicated by PCR band intensities (Fig. 4, second panel).

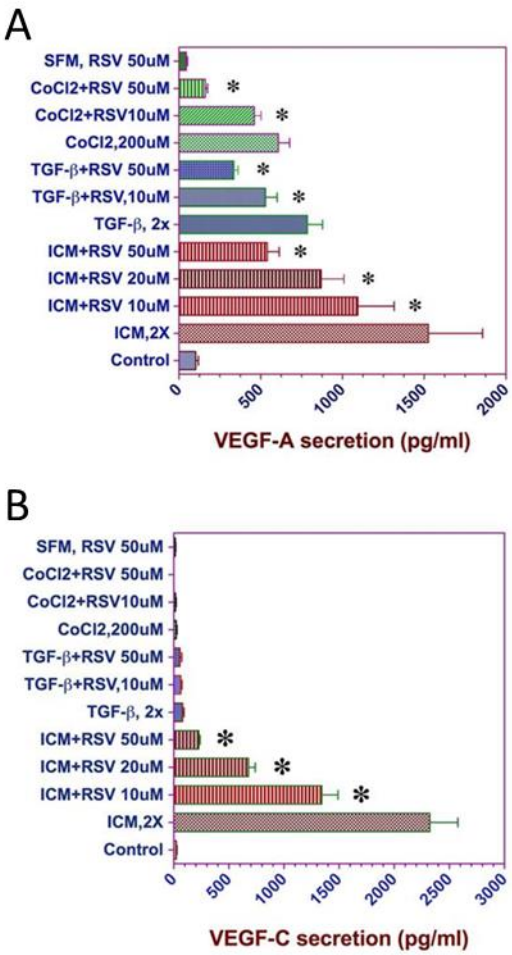

Figure 3. Effect of RSV on ICM, TGF-β and CoCl2 induced VEGF secretion by HRPE cells. Resveratrol inhibits ICM, TGF-β1 or hypoxia enhanced VEGF-A **(A)** and VEGF-C **(B)** secretion by HRPE cell. HRPE cells grown to confluence in 24 well plates were treated with ICM 2x (IFN-γ 20U +TNF-α 2ng + IL-1β 2ng/ml) alone or in the presence of various concentrations (10-50 uM) of RSV; TGF-β1 (2 ng/ml) alone or in the presence of various concentrations (10-50 uM) of RSV; CoCl2 (200 uM) alone or in the presence of RSV (10-50 uM) for 24h in serum-free media. Cobalt chloride (CoCl2) was used as a hypoxia mimic. Culture supernatants were collected and used for the determination of VEGF-A and VEGF-C levels by ELISA. TGF-β1 and Cobalt chloride (CoCl2) did not induce VEGF-C secretion by HRPE cells and RSV had no effect. Results are means ± SEM of 5 experiments each with duplicate samples. RSV effects compared to ICM, TGF-β or CoCl2, *P< 0.01-0.001

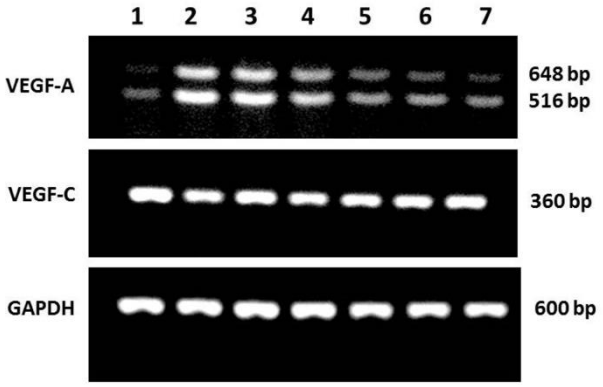

Figure 4. Resveratrol inhibits TGF-β1 enhanced VEGF-A but not VEGF-C mRNA expression by HRPE cells. Confluent cultures of HRPE grown in 60 mm plates were treated with TGF-β1 (2ng/ml) and in the presence of various concentrations (2-50 uM) of RSV for 8h in SFM. Total RNA prepared was used for RT-PCR analysis as described in the methods section. PCR amplification cycles were 25 for GAPDH and 30 for VEGF-A and VEGF-C. PCR products for VEGF-A, 516bp and 648 bp, represent VEGF-A proteins 121aa and 165aa respectively. PCR products in each group were exposed and integrated to the same intensity. Therefore comparisons of the band intensities between the groups are not appropriate. 1=control, 2= TGF-β1, 3= TGF-β1 +RSV (2 uM), 4= TGF-β1 +RSV (10 uM), 5= TGF-β1 +RSV (20 uM), 6= TGF-β1 +RSV (50 uM), 7=Control+RSV (20 uM).

Next, we examined the effects of ICM and RSV on physiologically relevant anti-angiogenic molecules, pigment epithelial derived factor (PEDF) and endostatin (fragment of collagen 18). Both PEDF and endostatin are constitutively secreted by HRPE in significant quantities (supplement Fig. C). ICM or combinations of ICM (2 x) and RSV (10-50 uM) did not alter PEDF and endostatin secretion (supplement Fig. C). The secretion of PEDF and endostatin was not affected by RSV alone (data not shown).

Finally, we wanted to explore the actions of RSV on HRPE cell proliferation and/or migration. A simple in vitro scratch wound assay was performed in either serum free medium (SFM) or in 5% FBS medium. Photomicrographs of HRPE cultures under different treatments are shown in Figure 5. HRPE cultures incubated in 5% FBS medium for two days after scratch treatment showed dense presence of cells in the wound area (compare Fig. 5 B to C). In the presence of RSV, dose dependent inhibition of cells in the wound area was evident (Fig. 5, D-F). Similar observations were made in experiments in which SFM was used instead of 5%FBS medium (data not shown). Further studies are needed to clarify whether the effects of RSV in wound closure assays were due to inhibition of cell migration or cell proliferation or both.

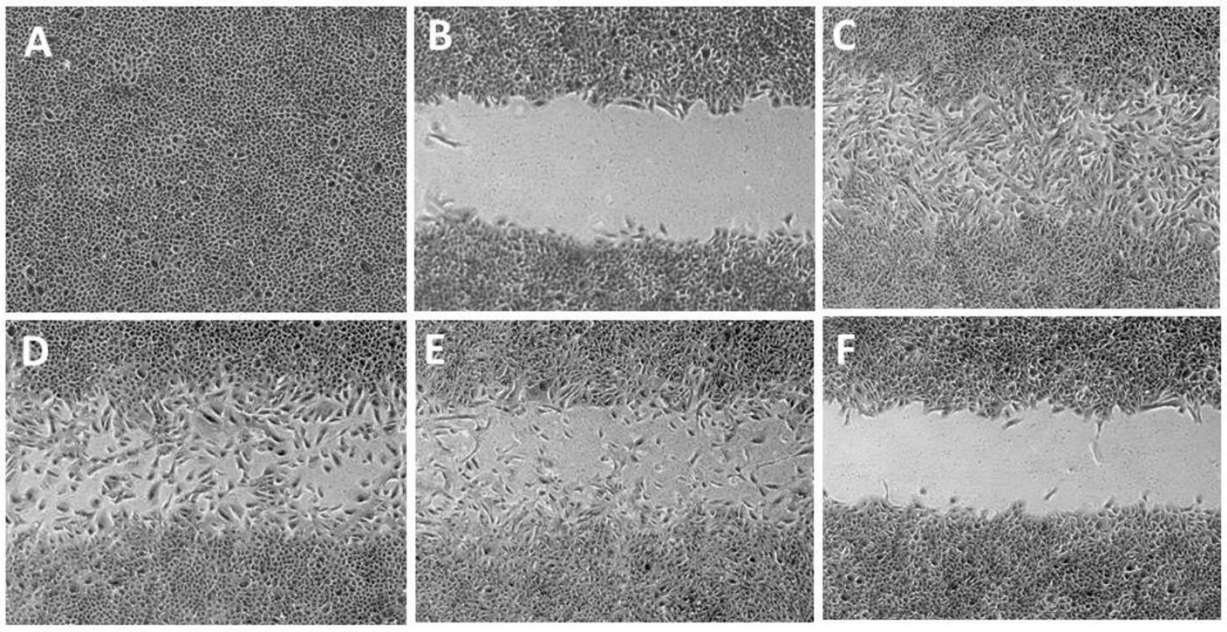

Figure 5. Effect of resveratrol on *in vitro* wound healing in HRPE cultures. HRPE cultures were grown to confluence in 8 well tissue culture glass slides. Using eppendorf microtip, uniform scratch was made in the center of the slide as described in the methods section. Cultures were washed and treated with various concentrations of RSV in a media containing 5% FBS. After 48 h incubation, cultures were washed, fixed, stained with GIEMSA stain and photographed. Representative photomicrographs are shown. A= control HRPE cultures; B=cultures after wounding, day 0; C= cultures treated with 5% FBS, day 2; D= cultures treated with 5%FBS+ RSV, 10uM, day2; E= cultures treated with 5% FBS +RSV, 20uM, day 2; F= cultures treated with 5%FBS +RSV, 50 uM, day 2.

DISCUSSION

Choroidal neovascularization in AMD is a serious retinal complication that is characterized by the growth of new blood vessels from existing choroid capillaries. These new vessels penetrate through breaks in Bruch's membrane (BM)-RPE into the photoreceptor layers [1, 4-9]. These processes result in abnormal and leaky blood vessels leading to fluid accumulation, macular edema, RPE degeneration and retinal detachments. This often results in loss of vision if not intervened therapeutically [1, 4, 7-9]. VEGF is one of the critical factors in the development and maintenance of blood vessels, and excess VEGF secretion is responsible for retinal vascular pathologies [7-9, 29, 30]. Therefore, current treatments for AMD are mainly centered on anti-VEGF therapies [26-28, 44]. Here, we show that RSV suppresses secretion of VEGF-A and VEGF-C produced by HRPE. Since HRPE cells are at the center of AMD pathology, RSV may be useful as a safe nutraceutical supplement for AMD patients in alleviating the AMD disease pathologies.

The exact pathways and causative factors associated with exudative AMD and associated CNV are not clear. Clinico-pathological studies of AMD retinal specimens point to inflammation and oxidative damage as likely candidates in the initiation and progression of the disease [44-48]. In the aging retina, extracellular deposits known as drusen accumulate between RPE-BM and RPE and photoreceptor layer [1, 5, 6, 48, 49]. Drusen contain complement factors, advanced glycation end products, lipids and inflammatory mediators. Drusen and oxidative damage products may be responsible for recruiting inflammatory cells such as macrophages, lymphocytes and neutrophils [48- 50]. In AMD pathological specimens, macrophages and lymphocytes are observed and they could be a source of inflammatory cytokines. Immunohistochemical studies demonstrated the presence of VEGF, PDGF, TGF-β and bFGF and their presence indicate a possible role in CNV [22-25, 51]. We have clearly shown that the inflammatory cytokine mix

(consisting of IFN-γ, TNF-α and IL-1β), TGF-β and hypoxia augment VEGF expression by HRPE cells [14, 18]. HRPE constitutively secrete both VEGF-A and VEGF-C in small quantities. This is essential for normal and healthy structure and functioning of choroid and retina [14, 18, 20, 29]. However, excess secretion of VEGF leads to vascular leakage and neovascularization.

A number of anti-VEGF therapeutic agents were developed to inhibit CNV and restore visual function [26-28, 44]. Treatments involve frequent intra-vitreal injections of anti-VEGF medications. These treatments are very expensive and are initiated after the appearance of late stage AMD characterized by the presence of CNV [26-28, 44]. Since the course of AMD disease development is slow and takes several years before the initiation of treatments, it will be very attractive to explore preventive measures. Several prospective, retrospective, randomized controlled trials and cross-sectional studies were conducted to find potential nutrients and other supplements that can delay or prevent AMD pathologies [52-55]. These long term studies revealed beneficial effects of anti-oxidants (vitamin C, E), carotenoids (lutein, zeaxanthin), omega-3 fatty acids and minerals (zinc, copper, selenium). Our present results, based on HRPE cell culture studies demonstrate potential beneficial effects of RSV which acts to inhibit vascular leakage and/or neovascularization by suppressing VEGF secretion.

In this HRPE cell culture study, we have shown that RSV inhibits inflammatory cytokine mix (ICM), TGF-β and hypoxia induced VEGF expression. In our previous report, we have demonstrated involvement of NFκB, JAK-STAT and MAP kinase pathways in the up regulation of VEGF-A and VEGF-C secretion by ICM or TGF-β [14, 18]. HRPE cells were also treated with lower concentrations of ICM to represent conditions that mimic in vivo pathophysiological conditions [6, 25, 45-48]. Even at lowest concentrations, ICM significantly enhanced VEGF secretion that was inhibited by RSV (Fig. 1). RSV inhibition of ICM induced VEGF-A and VEGF-C secretion could be due to suppressed gene transcription (Fig. 2). Oxidative damage, presence of drusen and RPE degeneration may create hypoxic microenvironment around RPE and enhances HIF-1α expression [6, 8, 48]. ICM and hypoxia treatment elevated NFκB and HIF-1α levels respectively in HRPE and RSV down regulated these transcription factors (data not shown), thereby inhibiting VEGF secretion. SIRT-1, a major modulator of RSV, represses HIF-1α signaling by deacetylation leading to reduced VEGF-A secretion [37, 38, 41, 42]. RSV was also shown to act via activation of eukaryotic elongation factor-2 kinase, SIRT-1 independent pathway, for inhibition of VEGF secretion, endothelial cell proliferation and migration [56]. Our in vitro scratch assay studies revealed that RSV prevented wound closure in a concentration dependent manner that could be due to cell migration, proliferation or both (Fig. 5).

RSV is a versatile molecule that mediates its effects by direct physical interaction with some proteins such as serum albumin, lipoproteins, amyloid fibrils and integrins as well as indirectly by modulating expression of several gene products [35-38]. RSV binds to a number of cell signaling molecules such as NFκB, STAT3, HIF-1α, PPAR γ, estrogen receptors and protein kinase C to regulate their functional activities [37, 38]. The expression of numerous gene products associated with inflammation, angiogenesis, metastasis, apoptosis, cell cycle and antioxidant pathways are regulated by RSV [37-42]. These activities include suppression of antiapoptotic gene products (BCL-2, survivin), induction of antioxidant enzymes (superoxide dismutase, hemeoxygenase-1), suppression of expression of inflammatory markers (COX2, iNOS, CRP, TNF-α). In HRPE cells, in addition to its anti-angiogenic activity by inhibiting VEGF expression, RSV acts as anti-oxidative damage and anti-inflammatory agent by regulating gene expression of several molecules [57, 58].

The polarized secretion of VEGF by retinal pigment epithelium was studied using ARPE-19 (immortalized RPE cells derived from eyes of 19 year old male) and HfRPE (primary RPE cells prepared from eyes of human fetus,15-20 weeks age) [59-63]. In ARPE-19 cells, predominant secretion of VEGF-A and VEGF-C towards apical side, that faces photoreceptor layer of neuroretina, was observed [59, 62]. In HfRPE cells, polarized secretion of VEGF-A was reported to be predominantly either apical or basal side that faces posterior choroid tissue [60-63]. It would be interesting to examine polarized secretion of VEGF-A and VEGF-C by HRPE cells used in the present studies. In any case, inhibition of induced VEGF secretion by RSV would be useful in controlling vascular leakage and neovascularization in chroidal tissue facing basal side of RPE as well as neuroretina facing apical side of RPE [10-12]. Thus beneficial effects of RSV include protection against pathological angiogenic processes both in choroidal tissue and neuroretina.

Elevated levels of VEGF are produced locally within the retina [6, 8, 21-25, 51]. CNV is the result of imbalance between angiogenic and anti-angiogenic molecules within the microenvironment of choroid-RPE-retina in the posterior part of the eye [21-25, 51]. Our studies have shown that RSV suppressed inflammatory cytokine mix, TGF-β and hypoxia enhanced VEGF-A and VEGF-C secretion by HRPE without influencing anti-angiogenic endostatin and PEDF secretion. Moreover, RSV did not affect basal secretion of VEGF-A and VEGF-C, both of which are critical for maintenance of healthy blood vessels in the choroid and retina [20, 21]. In addition to

anti-CNV effect, RSV is well known to exhibit anti-inflammatory, anti-oxidant and cardio-protective activities [57, 58]. RSV is also shown to protect heart, brain, kidney and other organs from ischemic reperfusion injury [37, 38, 40]. RSV enriched natural products and plant products containing RSV have been consumed for ages and have been reported to have no toxic effects [36-38, 64-66]. Therefore, it may be safe to consume RSV and RSV containing natural products, for example red grapes or grape extracts, at moderate doses. Based on these advantageous effects of RSV, we suggest that RSV would be useful as nutritional supplement by itself or as an adjunct to anti-VEGF therapies for maintaining RPE function and in controlling macular edema, diabetic retinopathy and CNV in AMD.

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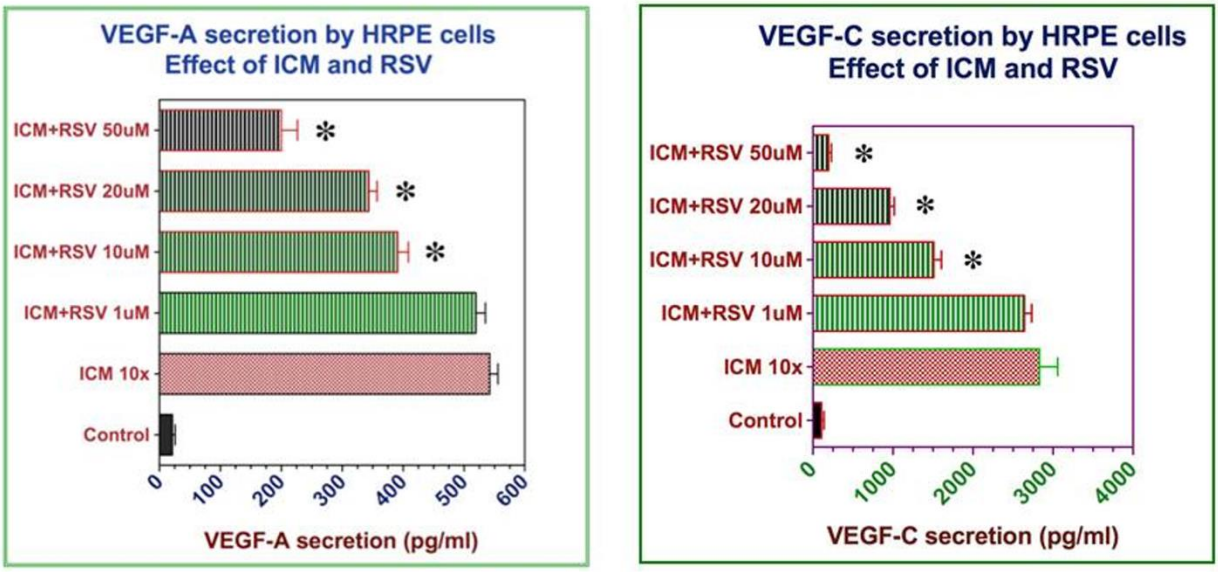
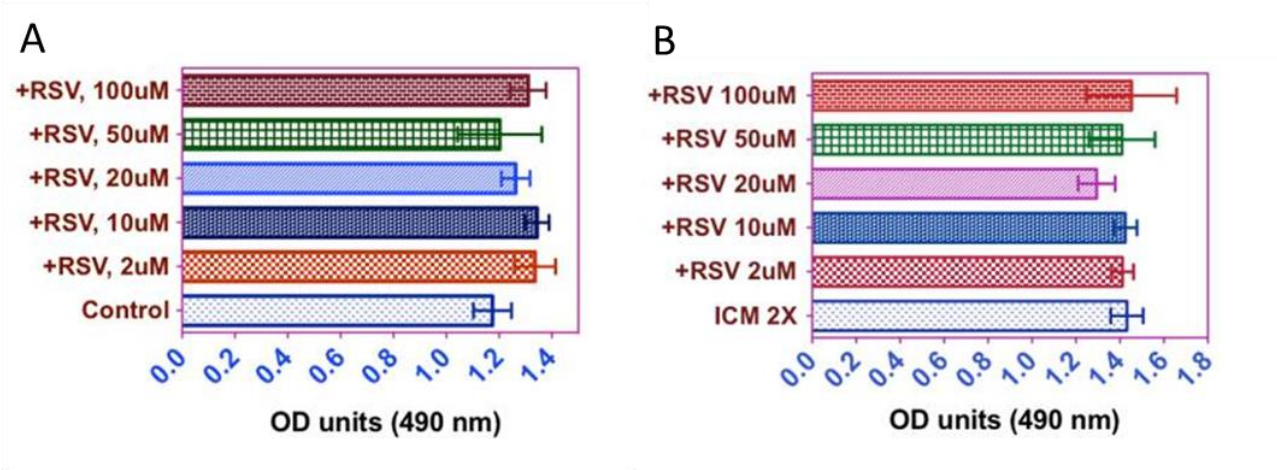
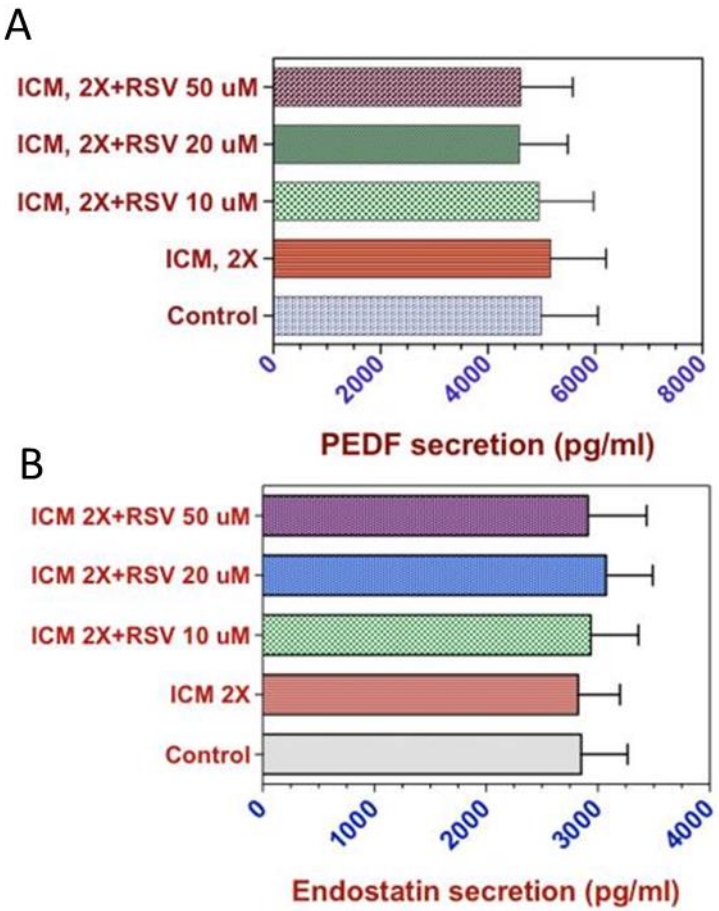
Supplemental Data



Supplemental Figure A. **Resveratrol (RSV) inhibits inflammatory cytokine mix (ICM) induced VEGF-A and VEGF-C secretion by human retinal pigment epithelial cells (HRPE).** Confluent HRPE cultures grown in 24 well plates were washed with serum-free medium (SFM) twice. Then SFM was removed and fresh SFM (1 ml/well) containing ICM 10x (IFN-γ, 100 U/ml), TNF-α (10 ng/ml) or IL-1β (10 ng/ml) in the presence of indicated concentrations (1-50uM) of RSV. After 24h incubation, culture supernatants were collected and used for the determination of VEGF-A and VEGF-C levels by ELISA. Results are means ± SEM of 5 experiments each with duplicate samples. Effect of RSV compared to ICM 10x, * P< 0.01-0.001.



Supplmental Figure B. HRPE cell viability was not affected by RSV alone **(A)** or RSV in the presence of ICM **(B).** HRPE cultures grown to confluence in 96 well plates were treated with various concentrations (2-100 uM) of RSV alone or RSV in the presence of ICM 2x (20U IFN-γ+2 ng TNF-α + 2 ng IL-1β /ml) for 24h. Studies were conducted both in serum free and 5% serum containing media. Cell viability was assessed by using Cell Titer Aqueous One (Promega) reagent as described in the methods section. Viability was expressed as optical density (OD) units. Results are means ± SEM of 3 experiments each with quadruplicate samples.



Supplemental Figure C. Resveratrol had no effect on **(A)** pigment epithelial derived factor (PEDF) and **(B)** endostatin secretion by HRPE cells. HRPE cells grown to confluence in 24 well plates were treated with ICM 2x (IFN-γ 20U +TNF-α 2ng + IL-1β 2ng/ml) in the presence of RSV (10- 50 uM) in SFM. After 24h incubation, culture supernatant fluids were collected and the levels of endostatin and PEDF were determined by ELISA. Endostatin (fragment of collagen 18) and PEDF are secreted proteins with potent anti-angiogenic activity. Results are means ± SEM of 4 experiments each with duplicate samples.